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Effects of Phorbol Myristate Acetate, Phorbol Dibutyrate, Ethanol, Dimethylsulfoxide, Phenol, and Seven Metabolities of Phenol on Metabolic Cooperation between Chinese Hamster V79 Lung Fibroblasts

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16. ABSTRACT

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The discovery that phorbol ester tumor promoters inhibit metabolic cooperation (MC) between several cell types in vitro fostered the idea that this effect might be associated with tumor promoters in general. We are testing this hypothesis by assessing the effects of tumor promoters, selected metabolites, and other chemicals on MC between cocultivated mutant ($HGPRT^-$) and wild-type ($HGPRT^+$) V79 cells in reconstructed selection experiments. Correlation studies show that many structurally-diverse promoters inhibit MC in a dose-related fashion. More recent studies show that ethanol, a surface-active solvent with cocarcinogenic activity in humans, inhibits MC at high concentrations (10-20 mg/ml). Phenol, a weak promoter of tumors on mouse skin, has no effect on MC at concentrations ranging from 50 to 400 ug/ml. Five metabolites of phenol block MC, but at least two others appear to be inactive as complete promoters for mouse skin. These correlation studies suggest that inhibition of MC is promising as a short-term marker for the identification of tumor promoters. Additional studies, however, are required to further establish the specificity of the endpoint for this application.

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EFFECTS OF PHORBOL MYRISTATE ACETATE, PHORBOL DIBUTYRATE, ETHANOL, DIMETHYLSULFOXIDE, PHENOL, AND SEVEN METABOLITIES OF PHENOL ON METABOLIC COOPERATION BETWEEN CHINESE HAMSTER V79 LUNG FIBROBLASTS

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The effect of phorbol myristate acetate, phorbol dibutyrate, ethanol, dimethylsulfoxide, phenol, and seven metabolites of phenol on metabolic cooperation were assessed as a function of mutant cell recovery from populations of cocultivated hypoxanthine-guanine phosphoribosyl transferase-deficient mutant (HGPRT-) and wild-type (HGPRT+) Chinese hamster V79 lung fibroblasts. Phorbol myristate acetate and phorbol dibutyrate, two established tumor promoters, were potent inhibitors of metabolic cooperation. Ethanol and dimethylsulfoxide, solvents commonly used to prepare chemicals for testing, weakly inhibited metabolic cooperation. Phenol and phenylglucuronide had no effect on metabolic cooperation. Four oxidative metabolites (1,4-benzoquinone, catechol, hydroxyquinol and quinol) inhibited metabolic cooperation. Phenylsulfate weakly inhibited metabolic cooperation. Conversely, 2-methoxyphenol, a methylated derivative of catechol, appeared to enhance metabolic cooperation. These results generally

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2. Key words: benzoquinone, catechol, cell culture, dimethylsulfoxide, ethanol, hydroxyquinol, metabolic cooperation, 2-methoxyphenol, phenol, phenylglucuronide, phenylsulfate, phorbol dibutyrate, phorbol myristate acetate, quinol, tumor promotion.

3. Abbreviations: CAS, Chemical Abstracts Service; DMSO, dimethylsulfoxide; ETOH, ethanol; HGPRT, hypoxanthine-guanine phosphoribosyl transferase; HGPRT+, HGPRT-competent; HGPRT-, HGPRT-deficient; MC, metabolic cooperation; MC+, metabolic cooperation-competent; MC-, metabolic cooperation-deficient; MEM, minimum essential medium; PDBu, phorbol dibutyrate; PMA, phorbol myristate acetate; 6TG, 6-thioguanine; 6TG^r, 6-thioguanine-resistant; 6TG^s, 6-thioguanine-sensitive; V79/MC assay, Chinese hamster V79 lung fibroblast assay for metabolic cooperation.

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support the hypothesis that tumor promoters inhibit metabolic cooperation and illustrate the importance of considering metabolites when testing this hypothesis. The weak capacity of five metabolites of phenol to inhibit metabolic cooperation correlates with the weakness of phenol as a tumor promoter. Interpretation of these results is complicated because two metabolic cooperation-inhibiting metabolites (catechol and quinol) are nonpromoting when tested individually in the same assay where phenol shows promoting activity. Such metabolites may be incomplete (stage) promoters, and exposure to two or more may be required for a promoting effect. The significance of enhanced metabolic cooperation requires further investigation, particularly in relation to antipromoting effects.

INTRODUCTION

Since its discovery in rabbit skin (Rous and Kidd, 1941), multistage (initiation/promotion) carcinogenesis has been demonstrated in many organs, including liver (Peraino et al., 1971), colon (Reddy et al., 1978), bladder (Bryan and Springberg, 1966; Hicks et al., 1975), pancreas (Longnecker et al., 1981), mammary gland (Carroll and Khor, 1970; Hopkins et al., 1976; Aylsworth et al., 1984), stomach (Fukushima et al., 1974), and the respiratory tract (Witschi, 1983; Steele and Nettesheim, 1984). Recent studies (Slaga et al., 1980; Furstenberger et al., 1981) support Boutwell's finding (1964) that mouse skin tumor promotion is, itself, a multistage process. In addition, smoking (Hoffmann et al., 1976, 1983), ethanol consumption under some conditions (Lieber et al., 1979; McCoy and Wynder, 1979), and occupational exposure to asbestos (Selikoff et al., 1964, 1968) are implicated in multistage carcinogenesis in humans. These facts have stimulated increased interest in the role of cocarcinogens and tumor promoters in cancer etiology and in the development of short-term tests for their detection.

It was recently discovered that tumor-promoting phorbol esters inhibit MC (exchange of low molecular weight signals via gap junctions) between cultured mammalian cells (Murray and Fitzgerald, 1979; Yotti et al., 1979). The gap junction is the most common type of cellular junction and is considered to be a channel of communication. It permits the direct exchange of small, water soluble molecules between the cytoplasms of adjacent cells, and couples such cells electrically (exchange of inorganic ions) and metabolically (exchange of larger molecules). The upper molecular weight limit of about 1500 daltons for gap-junctional communication implies that coupled cells may share inorganic ions, sugars, amino acids, hormones, other small peptides, nucleotides and vitamins, but not macromolecular proteins, nucleic acids and polysaccharides (Alberts et al., 1983). It was discovered that the gap-junctional transfer of a nucleotide (phosphorylated 6TG) between coupled mammalian cells in culture was inhibited in

the presence of PMA, a potent tumor promoter (Hecker et al., 1964; Baird and Boutwell, 1971). Since this discovery, suppression of MC by many structurally-diverse tumor promoters has been shown primarily in assays based on the inhibition of nucleotide transfer between cultured animal cells (for reviews, see: Trosko et al., 1982; Trosko and Chang, 1984). Inhibition of MC by many diverse promoters suggests both a role for MC in tumor promotion and a short-term assay for identifying potential tumor promoters. If initiated cells are deficient in critical regulatory molecules, inhibition of MC might prevent the transfer of such molecules from neighboring cells, and thereby facilitate the clonal expansion of initiated cell populations (Yotti et al., 1979; Trosko et al., 1982; Trosko and Chang, 1984).

In this paper, we briefly describe the V79/MC assay and present test results for twelve chemicals. The described experiments were designed to further test the hypothesis that tumor promoters inhibit MC, and to further validate the V79/MC assay as a short-term test for tumor promoters. Some of the results presented here have been summarized in previous publications (Malcolm et al., 1983a, 1983b, 1985).

METHODS

Cell culture. Wild-type (HGPRT+, $6TG^s$) and mutant (HGPRT–, $6TG^r$) Chinese hamster V79 lung fibroblasts, obtained from Dr. James E. Trosko, Michigan State University, were used in all experiments. The standard medium was Eagle's MEM (GIBCO, 320-1090) supplemented 0.5% with 100X concentrate MEM vitamin solution (GIBCO, 320-1120) and 1% with the following components: 50X concentrate MEM amino acids (GIBCO 320-1130); 100X concentrate MEM nonessential amino acids (GIBCO, 320-1140); 100X concentrate MEM sodium pyruvate solution (GIBCO, 320-1360); 100X L-glutamine solution (GIBCO, 320-5030); 83.5 mg/ml sodium chloride solution (salt dissolved in 18 megaohm deionized water).

Stock cells were maintained in the standard medium supplemented 10% with fetal bovine serum (GIBCO, 200-6140) and without antibiotics. All assays were conducted in assay medium (standard medium supplemented 5% with fetal bovine serum and 0.4% with penicillin-streptomycin solution (GIBCO, 600-5140)). Cell cultures were grown in water-jacketed incubators under standard conditions (37°C, 5% CO_2 in air, saturated humidity). Stock cells were grown in Nunc 100 × 20 mm tissue culture dishes (GIBCO, 150350) and maintained in a logarithmic growth phase by subculturing thrice weekly. Cultures were initially and periodically demonstrated to be free of mycoplasma contamination by an independent testing laboratory.

Cytotoxicity Assay. Before assessing the effects of a test chemical on MC, preliminary cytotoxicity tests were conducted with mutant cells to estimate doses which permit at least 70% mutant cell survival in MC experiments (see Discussion section). Stock cultures of mutant cells were washed twice with Earle's balanced salt solution without magnesium or calcium (GIBCO, 310-4150), then dispersed with 0.5 ml per dish 1X trypsin solution (REHEIS, 0240-00) prepared by diluting a 40X concentrate 1:40 with

Earle's balanced salt solution. Tryptic action was stopped after 5-10 minutes with assay medium (4 ml per dish). Cells were pooled into a 15 ml centrifuge tube (Corning, 25310) and gently agitated to produce a uniform, single-cell suspension. Cell suspension density was determined from hemocytometer counts. The cell suspension was then diluted with assay medium to a final density of 62.5 cells per ml. After thorough mixing, 250 cells were inoculated by automatic syringe into each of a series of Nunc 60 × 15mm tissue culture dishes (GIBCO, 150288), 5 dishes per dose. Dishes were incubated four hours for cell attachment. The test chemical was then added, simultaneously with 10 μg/ml of the selective agent, 6TG, via a medium change. The solvent (ETOH) was equated in all dishes and generally did not exceed 0.5%. Cells were exposed 48 hours to the test chemical. The exposure medium was then replaced with fresh assay medium containing only 6TG. Incubation was continued 4-5 additional days for macroscopic colony development. Colonies were fixed in ETOH and stained with crystal violet.Colonies containing at least 50 cells were counted, either electronically (Artek Model 980 Colony Counter) or by hand. Cytotoxicity was determined by comparing macroscopic colony development in experimental situations against that in the solvent control.

MC Assay. An experimental protocol for the V79/MC assay, as well as discussions of assay theory, have been published (Trosko et al., 1981; Trosko and Chang, 1984). Briefly, each test situation consisted of 21 Nunc 60 × 15mm tissue culture dishes: 15 dishes contained co-cultivated mutant and wild-type cells for assessing effects on MC, and 6 dishes contained only mutant cells for a concurrent estimate of cytotoxicity. First, stock mutant cells were dispersed as described above and diluted to 25 cells/ml in a large volume of assay medium. Mutant cells were inoculated, 100 cells per dish, into all dishes to be used for cytotoxicity assessment. Stock wild-type cells were then similarly dispersed and pooled into a 50 ml centrifuge tube (Corning, 25330). After gentle agitation, the wild-type cells were diluted to 100,000 cells/ml with the remaining assay medium already containing 25 mutant cells/ml. After thorough mixing, 4 ml of this suspension were transferred by automatic syringe to all dishes designated for MC assessment. Each MC dish thus contained approximately 100 mutant and 400,000 wild-type cells. As in preliminary cytotoxicity tests, dishes were incubated four hours for cell attachment. The test chemical and 6TG (10 μg/ml) were then simultaneously added via a medium change, and incubation continued another 48 hours. The exposure medium was then replaced with fresh medium containing only 6TG. Incubation was continued an additional 4-5 days for macroscopic colony development. Colonies were fixed, stained and counted as described above.

The solvent (ETOH) was equated in all test situations and a concurrent solvent control was included within each experiment. A concurrent, single-dose, positive control (PMA at 1 ng/ml or PDBu at 10 ng/ml) was also included with each assay. The capacity of test chemicals to affect MC was estimated from the average number of macroscopic colonies developing (mutant cell recovery) in a test situation relative to that in the solvent control. Differences were considered significant if mutant cell

recovery differed by a factor of two or more. Mutant recovery was not corrected for cytotoxicity as previously described by Trosko et al. (1981). Composite response curves for cytotoxicity and mutant cell recovery were constructed by averaging two or more independent experiments. For a few doses, multiple experimental values were not available. In such cases, the available mean value and its error were scaled up or down relative to the average control value, depending on whether the control value for this experiment was below or above the average control value. Experimentally, three results were possible. Chemicals had no effect on MC if mutant recovery in any test situation was not significantly different from that in the solvent control. MC was inhibited if mutant recovery in any test situation was significantly greater than that in the solvent control. MC was apprently enhanced if mutant recovery in any test situation was significantly less than that in the solvent control. Test situations used to assess the effects of substances on MC were limited to those estimated to permit at least 70% relative survival.

Test Chemicals. Test chemicals are grouped by manufacturer or supplier. Each substance is followed by its Chemical Abstract Service (CAS) registry number, supplier's catalog number, and the manufacturer's stated purity. Aaper Alcohol and Chemical Co., Louisville, KY (Ethyl alcohol, 64-17-5; absolute 200 proof). Aldrich Chemical Co., Milwaukee, WI (Catechol, 120-80-9, 13,501-1, 99+%; Hydroxyquinol, 533-73-3, 17,340-1, 99%; Quinol, 123-31-9, 24,012-5, 99+%; Phenol, 108-95-2, 18,545-0, 99+%). Fluka Chemical Corp., Hauppauge, NY (1,4-Benzoquinone, 106-51-4, 12309, 99+%; 2-Methoxyphenol, 90-05-1, 50880, 98%). Midwest Research Institute, Kansas City, MO (Potassium phenylsulfate, 1733-88-6, custom synthesis, lot 81-245-57, 98+%; Phenylglucuronide (Phenyl-β-D-glucopyranosiduronic acid), 17685-05-1, custom synthesis, lot 81-245-88, 98+%). Sigma Chemical Co., St. Louis, MO (Phorbol myristate acetate, 16561-29-8, P8139, purity not stated; Phorbol dibutyrate, 61557-88-8, P1269, purity not stated; 6-Thioguanine, 154-42-7, A4882, 98%). Pfaltz and Bauer, Waterbury, CT (Dimethylsulfoxide, 67-68-5, M28310, 99%). GIBCO, Grand Island, NY (Sodium chloride, 7647-14-5, 130830, biological grade).

RESULTS

The effects on MC between cultured V79 cells are shown for two common solvents (Fig. 1), two tumor-promoting phorbol esters (Fig. 2), and phenol and several identified major and minor metabolites of phenol (Fig. 3). ETOH and DMSO significantly inhibited MC at noncytotoxic but high (mg/ml) concentrations and were thus weak inhibitors of MC. PMA and PDBu induced almost complete mutant recovery at very low (ng/ml) concentrations. Phenol and phenylglucuronide showed no effect on MC whereas four minor oxidative metabolites (1,4-benzoquinone, catechol, hydroxyquinol and quinol) inhibited MC. Phenylsulfate weakly inhibited MC. One metabolite, 2-methoxyphenol, appeared to enhance MC.

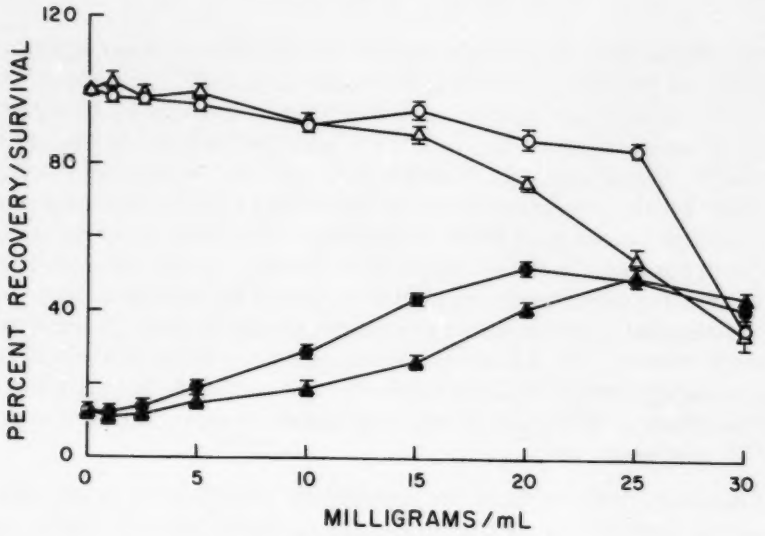

FIGURE 1. Dose-responses for DMSO (o) and ETOH (Δ). Open symbols represent percent relative survival estimated from cytotoxicity tests with mutant cells. Closed symbols represent mutant cell recovery resulting from effects on MC between mutant and wild-type cells. Curves were constructed from mean values plotted with their standard errors.

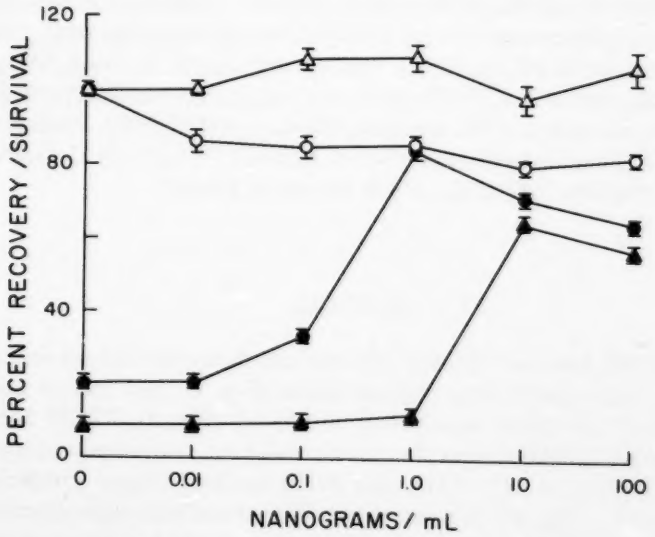

FIGURE 2. Dose-responses for PMA (o) and PDBu (Δ). Data treatment and representation are as in Figure 1.

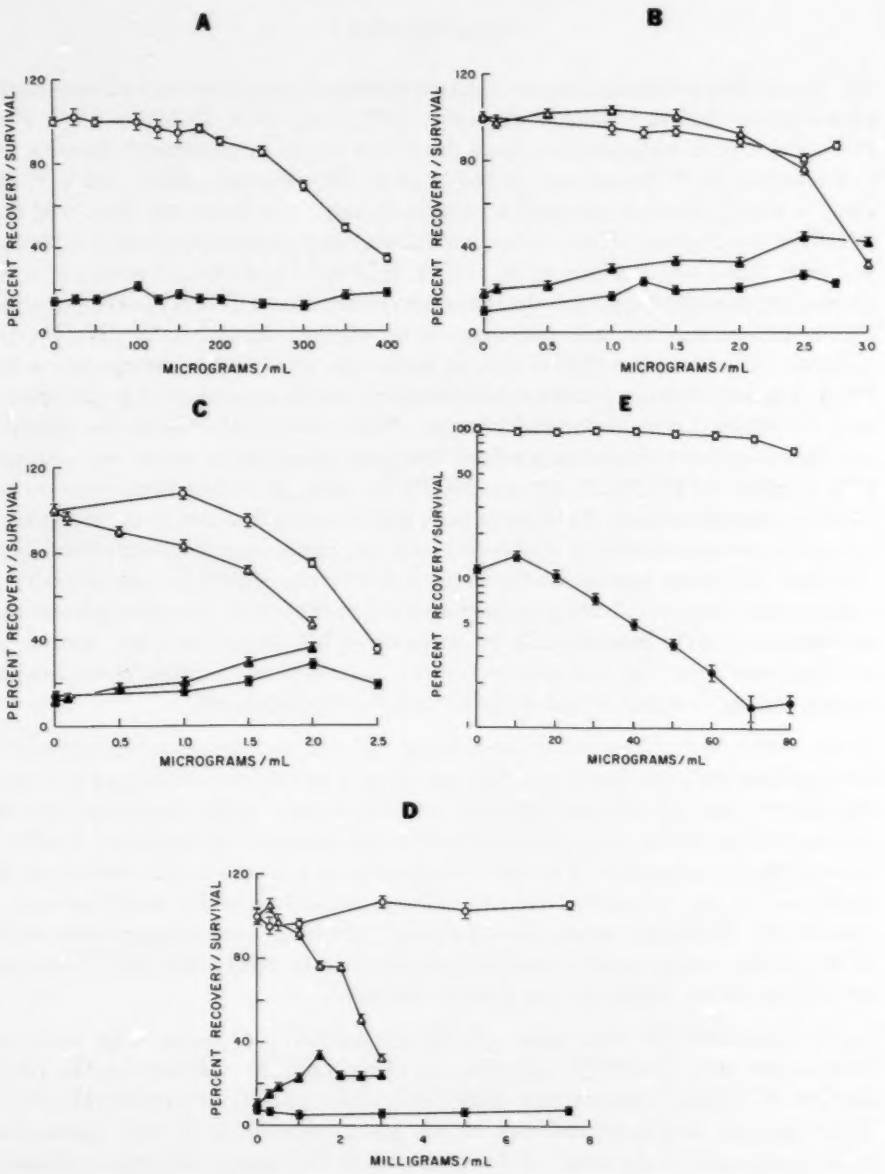

FIGURE 3. Dose-responses for phenol and seven metabolites of phenol. Data treatment and representation are as in Figure 1, except for 2-methoxyphenol, in which case the dose-response plot is semilogarithmic to better illustrate the decline in mutant survival. A, phenol; B, 1,4-benzoquinone (o) and catechol (Δ); C, hydroxyquinol (o) and quinol (Δ); D, phenylglucuronide (o) and potassium phenylsulfate (Δ); E, 2-methoxyphenol.

DISCUSSION

MC Assay. Gap-junctional communication between animal cells is a well-established phenomenon (Subak-Sharpe et al., 1966, 1969; Cox, 1974; DeMello, 1977; Pitts, 1980) and several methods have been developed for its measurement (Socolar and Loewenstein, 1979; Hooper and Subak-Sharpe, 1981; Hooper, 1982). The V79/MC assay is based on toxic nucleotide transfer through gap junctions from wild-type (HGPRT+) to mutant (HGPRT–) cells in cocultivated populations (Subak-Sharpe et al., 1966, 1969; van Zeeland et al., 1972). HGPRT– mutants are deficient in the enzyme hypoxanthine-guanine phosphoribosyl transferase (HGPRT) which catalyzes the conversion of purine bases into their corresponding ribonucleotides. HGPRT also catalyzes the conversion of 6TG into its nucleotide which, upon incorporation into DNA, is lethal. Because plasma membranes are usually impermeable to phosphorylated molecules (Leibman and Heidelberger, 1955), nucleotides become incorporated into the nucleic acid of those cells within which phosphorylation occurs. Accordingly, 6TG is lethal to HGPRT+ but not HGPRT– cells, providing these cells do not establish physical contact. If contact occurs, gap junctions form and toxic, phosphorylated 6TG is transferred from wild-type to mutant, resulting in the death of both cells. This MC-dependent interaction between HGPRT+ and HGPRT– cells provides an experimental basis for identifying chemicals which effect MC. Compounds reducing the transfer of 6TG between cells by suppressing MC should increase mutant cell survival relative to that in a solvent control. Alternatively, substances may reduce mutant cell survival below that in the control by enhancing MC.

Cytotoxicity Assay. Toxicity tests conducted with only mutant cells at low density do not duplicate the experimental conditions under which effects on MC are measured (100 mutant and 400,000 wild-type cells per 60 mm dish). Such toxicity tests provide only an estimate of the cytotoxicity present in MC assays. The absence of HGPRT+ cells eliminates facilitation of mutant cell attachment and growth via medium conditioning effects, and increases the amount of toxicant per cell, with possible increases in cytotoxicity. Therefore, the current cytotoxicity test likely overestimates cytotoxicity in MC assays except in those situations (believed to be rare) where the V79 cell can metabolize the test agent to a more toxic substance.

Other approaches for estimated cytotoxicity are being explored. One technique involves the use of HGPRT– cells that are also genetically deficient for MC (Aylsworth et al., 1984). In the presence of HGPRT+ cells and 6TG, survival of (HGPRT–, MC–) mutants should depend only on test agent cytotoxicity. If true, cytotoxicity could be assessed at the same cell densities used in MC assays. Extensive validation will be required to ensure that (HGPRT–, MC–) mutants respond in the same way to test substances as the (HGPRT–, MC+) mutants actually used in MC assays.

PMA, PDBu. PMA and PDBu are routinely used as positive controls in MC assays. The capacity of PMA and PDBu to inhibit MC correlates with their potency as mouse skin tumor promoters (Hecker et al., 1964; Baird and Boutwell, 1971; Slaga et al.,

1976, 1981). Our results are similar to those reported by others (Yotti et al., 1979, 1981; Murray and Fitzgerald, 1979; Dorman and Borieko, 1983). In addition to promoting tumor development on mouse skin, PMA also promotes lung tumor development in hamsters (Sellakumar et al., 1975) and tracheal carcinogenesis in rats (Topping and Nettesheim, 1980).

ETOH, DMSO. ETOH is epidemiologically implicated as a cocarcinogen or tumor promoter in humans (Lieber et al., 1979; McCoy and Wynder, 1979), and is predicted to have tumor-promoting properties because of its surface activity (Boyland, 1983). Like other anesthetics, ETOH has major effects on cell membranes. ETOH is reported to intercalate between the fatty acids of membrane phospholipids, inducing membrane expansion and increased fluidity (Seeman, 1972). ETOH is also reported to inhibit Na^+/K^+-dependent and Ca^{+2}-dependent ATPases (Burke et al., 1977) which maintain normal cellular Na^+/K^+ balance and low intracellular free calcium levels (Alberts et al., 1983). Indeed, intracellular calcium mobilization coincides with inhibition of MC (Loewenstein, 1981) and other cellular events associated with tumor promotion (Diamond et al., 1980; McGaughey and Jenson, 1980; Weinstein et al., 1982; Flavin and Kolbye, 1983).

DMSO was less cytotoxic than ETOH and a more potent inhibitor of MC. Similar responses for DMSO in the V79/MC assay are reported by Dorman and Boreiko (1983) and Chen et al. (1984). The tumor-promoting properties of DMSO do not appear to have been investigated. Although DMSO strongly inhibits mouse skin tumor promotion by PMA (Slaga et al., 1981), other promoters such as butylated hydroxytoluene (Peraino et al., 1977; Witschi, 1983) and acetic acid (Slaga et al., 1975) also inhibit promotion on mouse skin by PMA (Slaga et al., 1981, 1983). Because DMSO is a solvent for PMA, inhibition of PMA-induced promotion by DMSO may be due to the rapid absorption of DMSO (and PMA) through the skin (David, 1972), or to the effectiveness of DMSO as a scavanger (Fox and Fox, 1983) of potentially promoting oxygen radicals (Troll et al., 1982). In V79 cells, DMSO has no effect on the capacity of PMA to suppress MC (Malcolm and Mills, unpublished data). DMSO induces membrane effects which may increase intracellular free calcium (Shlafer, 1975; The and Hasselbach, 1977) and also induces differentiation in Friend leukemia cells, further suggesting the cell membrane as an important site of action (Tapiero et al., 1983). The high concentration of DMSO and ETOH required for inhibition of MC suggest osmotic effects which might disrupt gap junctional communication by causing cells to round up (Dorman and Borieko, 1983) or to decrease in size.

Phenol and Its Metabolites. Phenol is a weak promoter of tumors on mouse skin (Boutwell and Bosch, 1959; Van Duuren et al., 1978). When phenol was found to have no effect on MC, even at cytotoxic concentrations, several metabolites were tested because of the importance of skin as a drug-metabolizing organ (Pannatier et al., 1978) and because the action of some promoters may be mediated through metabolites. Inhibition of MC by five metabolites of phenol indicates that phenol is not an exception to the hypothesis that promoters inhibit MC, and demonstrates the need to

consider metabolites when testing this hypothesis. In addition, the relative weakness of these metabolites as inhibitors of MC correlates with the weakness of phenol as a tumor promoter (Boutwell and Bosch, 1959). Inhibition of MC by the minor oxidative metabolites (benzoquinone, catechol, hydroxyquinol and quinol) occurred at concentrations (1-3 μg/ml) which could be achieved in tissues as a result of metabolic activity. It is unlikely, however, that the high concentration (mg/ml) of phenylsulfate required to inhibit MC would occur in tissues, despite the fact that this product is a major metabolite of phenol (Williams, 1964).

Neither catechol nor quinol is promoting when tested individually in the same assay where phenol shows promoting activity (Boutwell and Bosch, 1959). One explanation may be that mouse skin tumor promotion is multistage (Boutwell, 1964; Slaga et al., 1980; Furstenberger et al., 1981) and that some incomplete (stage) promoters effectively inhibit MC. For example, the ionophore A23187, 4-0-methyl-PMA, and wounding are classified by Slaga (1984) as stage I promoters in mice. Mezerein is classified primarily as a stage II promoter, whereas hydrogen peroxide is reported to be both a stage I and a stage III promoter of mouse skin tumors. Inhibition of MC by wounding and A23187 correlates with increases in intracellular free calcium (Loewenstein, 1972; Flagg-Newton and Loewenstein, 1979). Inhibition of MC by hydrogen peroxide (Cox et al., 1974), 4-0-methyl-PMA and mezerein (Trosko et al., 1982) may also be accompanied by mobilization of intracellular free calcium. Thus, the suppression of MC by stage promoters could have mechanistic implications for the non-promoting but MC-inhibiting metabolites of phenol. If these are also stage promoters, exposure to two or more might be required for tumor promotion. The fact that the stage II promoter, mezerein, is almost as effective an inhibitor of MC as PMA, suggests that inhibition of MC alone is insufficient for tumor promotion. Recent studies by Kalimi and Sirsat (1984) suggest the relevance of gap junctions to stage I tumor promotion in mouse epidermis.

Enhanced MC. In the current assay, no exogenous metabolizing system is used. Because the V79 cell has a limited capacity to metabolize many chemicals (Bradley et al., 1981), observed responses are assumed to result from the effects of unmetabolized test substances. 2-Methoxyphenol thus appears to enhance MC. Enhanced MC could occur through a stimulated increase in the number of gap junctions. Retinoic acid, an antipromoter under some conditions, increases the number of gap junctions on the membranes of some cells (Elias et al., 1980; Larsen, 1977), thereby increasing the opportunity for MC. Also, chemicals causing cells to increase in size could enhance MC by increasing the probability of cell contact. If, however, 2-methoxyphenol were metabolized by the V79 cell to a more toxic substance, mutant recovery might decrease with increasing dose as shown in Figure 3E. This phenomenon would not generally be detected in the current toxicity assay as 250 cells per dish would not generate sufficient metabolite for measurable effects; however, in MC assays with 400,000 cells per dish, enough metabolite for measurable effects might be produced. Thus, the conclusion that 2-methoxyphenol enhances MC is provisional. In summary

our results generally support the hypothesis that tumor promoters inhibit MC and demonstrate the need to consider metabolites when testing this hypothesis. The indication that chemicals can enhance as well as inhibit MC requires further investigation, particularly in regard to possible antipromoting effects. Inhibition of MC by two nonpromoting metabolites of phenol suggests that these substances may act as incomplete (stage) promoters. The effective inhibition of MC by several established stage promoters suggests that inhibition of MC alone is insufficient for tumor promotion. In fact, the large number of cellular events involved in the promotion process suggests that many such events will be necessary or facilitating but not sufficient for tumor promotion. The role of MC in tumor promotion and its suppression as a short-term test for promoter identification deserve further study.

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